a) contacting a nucleic acid sample with a DNA binding domain of a restriction endonuclease that cleaves said nucleic acid sample 300,000 times for fewer, wherein said sample comprises a subset of nucleic acid molecules having a sequence that is bound by said DNA binding domain, and wherein a bound subset of nucleic acid molecules is retained by said DNA binding domain, such that the subset of bound nucleic acid molecules is enriched for molecules comprising the sequence recognized by said DNA binding domain; and

b) detecting a sequence difference with respect to a reference sequence in the subset of nucleic acid molecules,

wherein steps (a) and (b) enrich for and identify a nucleic acid sequence difference with respect to a reference sequence.

158. (New) The method of claim 157 wherein said restriction endonuclease that cleaves said nucleic acid sample 300,000 times or fewer is selected from the group consisting of AscI, BssHII, EagI, NheI, NotI, PacI, PmeI, RsrII, SalI, SbfI, SfiI, SgrAI, SpeI, SrfI, and SwaI restriction endonucleases.

REMARKS

Claims 1-5, 57-74 and 145-156 are pending. Claims 1, 57 and 69 are amended, and new claims 157 and 158 are added.

New claims 157 and 158 find support on page 4, line 10 to page 5, line 5 and on page 31, lines 5-13.

Rejections under 35 U.S.C. §112, second paragraph:

Claims 1-3, 57-74 and 145-156 are rejected under 35 U.S.C. §112, second paragraph for alleged indefiniteness.

Claims 1-3, 57-74 and 145-156 are alleged to be indefinite for lack of a final process step that clearly relates back to the preamble. Applicants submit that the amendment of claims 1, 57 and 69 are sufficient to overcome this basis of rejection. Claim 1 is amended to recite, after step (b), "wherein steps (a) and (b) enrich for and identify a nucleic acid sequence difference with

respect to a reference sequence." Claim 57 is amended to recite, after step (e), "wherein steps (a)-(e) enrich for and identify a nucleic acid sequence difference with respect to a reference sequence." Claim 69 is amended to recite, after step (c), "wherein steps (a)-(c) enrich for and identify a nucleic acid sequence difference with respect to a reference sequence." Applicants submit that each of these claims as amended clearly relates the outcome of the recited steps back to the preamble of the claim. Because claims 2, 3, 58-68, 70-74 and 145-156 all depend from one of amended claims 1, 57 or 69, these claims also clearly relate the outcome of the recited steps back to the preamble of the claim. Applicants therefore request the withdrawal of this rejection of claims 1-3, 57-74 and 145-156 under §112, second paragraph.

Claims 57-74 are alleged to be indefinite for use of the term "capable of" in independent claims 57 and 69. Applicants submit that the amendments herein of claims 57 and 69, removing the "capable of" language, are sufficient to overcome this rejection.

In claim 57, the "molecules capable of being replicated" language has been amended to "an oligonucleotide or a vector." The linkage to an "oligonucleotide or a vector" is supported in the specification at page 30, lines 10-11, page 64, lines 9-14, page 74, line 12 to page 76, line 19, and page 77, lines 9-15.

In view of the amendments to claims 57 and 69 that remove the "capable of" language, Applicants respectfully request the withdrawal of this §112, second paragraph rejection of amended claim 57 and claims 58-68 that are dependent from it, and of amended claim 69 and claims 70-74 that are dependent from it.

Claims 155 and 156 are rejected as allegedly indefinite under §112, second paragraph for use of the term "infrequently." The Office Action states that "[i]t is not clear what kind of infrequency is claimed in this invention." The Office Action concludes that the term is "relative, vague and indefinite," rendering the metes and bounds of the claim vague and indefinite. Applicants respectfully disagree.

The term "infrequently" as used in claims 155 and 156 is defined in the specification on page 31:

> "As used herein, the term □infrequently,□ as applied to cleavage of mammalian DNA (e.g. human DNA) by a restriction endonuclease refers to cleavage which occurs 300,000 times or less in a given genome (for example, 250,000, 200,000, 150,000, or 100,000 times) or which generates an average fragment size of 10,000 bp or more (for example, 20,000 bp, 30,000 bp, 50,000 bp) when a given genomic DNA sample is digested. These frequencies are particularly applicable to human DNA." (page 31, lines 5-10).

In view of this definition, Applicants submit that there is nothing vague, relative or indefinite about the term "infrequently" in claims 155 and 156. In those claims, the term is clearly applied to the cleavage of DNA by a restriction endonuclease. Therefore, in claims 155 and 156, "infrequently" means that the restriction endonuclease cleaves mammalian DNA 300,000 times or less in a given genome or generates an average fragment size of 10,000 bp or more when a given genomic DNA sample is digested. Applicants therefore request withdrawal of the §112, second paragraph rejection of claims 155 and 156 over the use of this term.

Rejections under 35 U.S.C. §102:

Claims 1-3, 57-74 and 150-153 are rejected under 35 U.S.C. §1-2(a) for alleged anticipation by Oefner et al. (U.S. Patent No. 5,795,976). The Office Action states that Oefner et al. teaches "a method of enriching for and identifying a nucleic acid sequence difference with respect to a reference sequence comprising (a) hybridizing a nucleic acid sample with a nucleic acid molecule comprising a sequence-specific binding activity under conditions which permit specific binding, wherein the sample comprises a subset of nucleic acid molecules having a sequence that binds to the sequence-specific binding activity, and wherein a bound subset of nucleic acid molecules is retained by the sequence-specific binding activity, such that the subset of bound nucleic acid molecules is enriched for molecules comprising the sequence recognized by the sequence-specific binding activity," and "(b) detecting a sequence difference with respect to a reference sequence in the subset of nucleic acid molecules." On this basis, the Office Action concludes that claim 1 is anticipated by Oefner et al. Applicants respectfully disagree.

First, Oefner et al. does not teach a method in which a sample comprises a subset of nucleic acid molecules having a sequence that binds to a sequence-specific binding activity as required by claim 1. As defined in the specification, the term "subset of molecules" refers to that fraction of a population of nucleic acid fragments, *less than every molecule in the population*,

having a given characteristic (e.g., having ends capable of annealing to a particular linker or primer, or having a particular average length)" (emphasis added; page 29, line 20 to page 30, line 2). In the method taught by Oefner et al., there is no subset of nucleic acid molecules having a sequence that binds to a sequence-specific binding activity, because *all* of the nucleic acid in a sample can bind to the sequence-specific binding activity. In the method of Oefner et al., some nucleic acids in a sample bind as homoduplexes (no mismatches) and some nucleic acids bind as heteroduplexes (at least one mismatch), but all of them bind to the same sequence-specific binding activity, so one cannot say that there is a "subset" of nucleic acid molecules that binds the sequence-specific binding activity. Therefore, Oefner et al. does not teach the method of claim 1 or claims that depend from it.

The Office Action also states that Oefner et al. teaches a method of enriching for and identifying a nucleic acid sequence difference with respect to a reference sequence, comprising a) fragmenting a nucleic acid sample from one or more individuals (citing column 9, lines 39-43); b) physically separating a subset of the nucleic acid fragments based on the size of the fragments (citing Example 2 and column 13, line 21 to column 17, line 12); c) operatively linking the subset of step (b) with molecules capable of being replicated (citing column 13, line 21 to column 17, line 12); d) introducing the linked subset of molecules of step (c) into a system capable of replicating the linked subset of molecules, and replicating the subset of linked molecules to form an enriched collection of replicated molecules (citing column 17, lines 14-67); e) detecting one or more nucleotide differences in the members of the collection of step (d) with a method capable of detecting one or more nucleotide differences with respect to a reference sequence (citing column 18, lines 1-30 and Example 7, column 34, lines 7-13, Example 8, column 34, line 53 to column 36, line 27 and Figures 11A and 11B). On this basis, the Office Action concludes that Oefner et al. anticipates claim 57. Applicants respectfully disagree.

Applicants wish to point out that the language of claim 57 specifies an order in which the steps comprising the method are performed in order to result in the enrichment for and identification of nucleic acid sequence differences with respect to a reference sequence. Step (a) requires fragmenting a nucleic acid, step (b) requires physically separating a subset of *those* fragments ("said nucleic acid fragments"), step (c) requires operatively linking "said subset of step (b)," step (d) requires "introducing the linked subset of step (c)," and step (e) requires

detecting one or more nucleotide sequence differences "in the members of said collection of step (d)." The Office Action has cited text in the Oefner et al. specification that the Examiner construes as describing the individual steps of the claimed method, but those steps are not taught or suggested to be in the order required by claim 57. Applicants submit that this piecemeal arrangement of steps alleged to satisfy the requirements of claim 57 cannot satisfy these requirements unless they are also taught to be performed in the order required by the claim. Applicants acknowledge that claim 57 uses open "comprising" language in setting out the steps of the claimed method, but those steps are clearly connected, by their own language, in an order necessary for the method to produce the desired outcome. Applicants note that this connection in no way precludes the presence of additional method steps (e.g., a new step between steps (b) and (c)), but rather requires that in a method comprising, among others, steps (a) through (e), steps (a) through (e) must be present in the order presented in the claim.

The teachings construed by the Office Action to satisfy the recited steps of claim 57 do not support their assembly into the order required by claim 57. For example, the Office Action cites Example 2 and column 13, line 21 to column 17, line 12 for step (b), "physically separating a subset of said nucleic acid fragments based on the size of the fragments." Example 2 of Oefner et al. relates to the detection of homoduplexes with base overhangs by denaturing HPLC. This could possibly be construed as a "physical separation" of nucleic acid fragments based on the size of the fragments. However, Oefner et al. does not teach "operatively linking" either separated species generated in Example 2 to an oligonucleotide or a vector, nor does the reference teach a step of "replicating the operatively linked subset to form an enriched collection of replicated molecules" as required by claim 57 as amended. According to claim 57, "operatively linking" and "replicating" steps must necessarily follow the physical separation step. Because the teachings in Example 2 do not teach such steps following the alleged size separation step, the teachings of Example 2 do not anticipate claim 57.

Similarly, in column 13, line 21 to column 17, line 12 of Oefner et al., cited in the Office Action, there is no step of "physically separating a subset of said nucleic acid fragments based on the size of the fragments" followed by a step of "operatively linking" the subset of size-separated fragments to "an oligonucleotide or vector." If, for the sake of argument alone, one assumes that the PCR amplification of a polymorphic region of the Y chromosome described at column 15,

lines 1-13 is interpreted as replicating an operatively linked subset to form an enriched collection of replicated molecules, Applicants submit that this step is not preceded by any step of "physically separating a subset of fragments based on the size of the fragments," as required by claim 57. Applicants submit that the only step taught by Oefner et al. that might be construed as having a size separation element relative to a fragment that is replicated is the isolation of the Y chromosome PCR product, but this step is not specifically set forth, and even if it were, it *cannot precede* the "replicating" step of PCR. In the method described by Oefner et al., such a size-separating step *must necessarily follow* the replicating step. One cannot size separate a fragment one has not generated. Because this order does not comply with the order required by the language of claim 57, this teaching of Oefner et al. does not anticipate claim 57.

Because Oefner et al. does not anticipate claim 1 or claim 57, the reference does not anticipate claims dependent from them, including claims 1, 3, 58-68 and 150-153.

Applicants submit that Oefner et al. does not anticipate claim 69 as amended because Oefner et al. does not teach a step of "fragmenting a nucleic acid sample from one or more individuals." The Office Action cites column 9, lines 39-43 of the Oefner et al. reference as teaching the "fragmenting" step. Applicants submit that the cited passage is part of the definition of "comparative DNA sequencing" set forth by Oefner et al.:

> "'Comparative DNA sequencing' as used herein refers to a method for detecting any, and preferentially all, possible mucleotide mismatches and insertion/deletions within ***select*** amplified or non-amplified DNA fragments obtained from multiple animal or human subjects. In comparative DNA sequencing, DNA samples, typically up to at least about 1.5 kB in length, are obtained from multiple subjects and *amplified or otherwise produced (e.g., by cloning)*. The amplified DNA fragments are then surveyed, either individually or in pools containing up to about 10 unique samples, for the presence or absence of heteroduplexes." (Oefner et al., column 9, lines 39-49; emphasis added).

Taken in context, Applicants submit that Oefner et al.'s teaching of the analysis of "select" amplified or non-amplified DNA fragments does not involve "fragmenting," but rather, synthesis of select fragments by amplification or cloning. Because Oefner et al. does not teach the step of fragmenting a nucleic acid sample from one or more individuals, Applicants submit that Oefner et al. cannot be said to anticipate claim 69 or claims 70-74 that depend from it.

In view of the above, Applicants respectfully request the withdrawal of the §102(a) rejection of claims 1-3, 57-68, 69, 70-74 and 150-153 over Oefner et al.

Rejections under 35 U.S.C. §103:

A) The Oefner et al. reference in view of Bloch:

Claims 1-3, 57-74 and 145-155 are rejected under 35 U.S.C. §103 for alleged obviousness over Oefner et al. in view of Bloch et al. (U.S. Patent No. 5,866,429). The Office Action states that Oefner et al. does not teach fragmenting a nucleic acid sample by endonuclease digestion, and that Bloch et al. teaches fragmenting a nucleic acid sample by endonuclease digestion. The Office Action further states that Bloch et al. teaches the method wherein at least one restriction endonuclease cleaves DNA infrequently (citing Example 4, column 24, lines 5-8). The Office Action then concludes that it would have been "obvious to substitute and combine within the method of comparative hybridization and sequencing of Oefner et al., the method of restriction endonuclease digestion of Bloch et al., since Bloch et al. state 'Another preferred method, used alone or together with PCR, for providing nucleic acid suitable for HPLC analysis is digestion with a restriction endonuclease, a procedure which, for relatively homogeneous DNA, generates a finite and often low number of well defined fragments.'" Applicants respectfully disagree.

First, Bloch et al. does not teach a method wherein at least one restriction endonuclease cleaves DNA infrequently. As discussed above in relation to the term "infrequently," the definition of a restriction endonuclease that cleaves DNA infrequently requires that it generate an average fragment size of 10,000 bp or greater or that it cleaves a given mammalian genome 300,000 times or fewer. The restriction endonuclease HindIII, taught by Bloch et al. at the passage cited in the Office Action (column 24, lines 5-8), generates an average fragment size of about 4,000 bp upon cleavage of human DNA (New England Biolabs Catalog reference appendix; Exhibit A) and cleaves the human genome approximately 750,000 times (3×10^9 bp per human genome/4,000 bp). Bloch et al. therefore does not teach a method using at least one restriction endonuclease that cleaves DNA infrequently.

Second, Applicants submit that the combination suggested by the Office Action does not result in the invention as claimed in claim 1 or claims dependent from it. Bloch et al. does not remedy Oefner's lack of a teaching that the sample comprises a subset of nucleic acid molecules having a sequence that binds to the sequence-specific binding activity. In the case in which one assumes, strictly for the sake of argument, that Oefner et al. teaches nucleic acid as a sequence-specific binding activity, Bloch et al.'s teaching of using a restriction endonuclease to generate fragments for subsequent analysis by HPLC does not remedy Oefner's deficiency with regard to claim 1 because the restriction endonuclease does not make it so that a subset of nucleic acid molecules is retained by the sequence -specific binding activity. Rather, all of the fragments, when mixed, denatured and allowed to anneal according to the method of Oefner et al., will still bind the nucleic acid sequence-specific binding activity (as either a homoduplex or a heteroduplex), meaning that there is still no subset having a sequence that binds to the sequence-specific binding activity.

Alternatively, if one assumes, again, for the sake of argument, that the restriction endonuclease taught by Bloch et al. is the sequence-specific binding activity, the sequence-specific binding activity does not satisfy the claim element "wherein a bound subset of nucleic acid molecules is retained by the sequence-specific binding activity, such that the subset of bound nucleic acid molecules is enriched for molecules comprising the sequence recognized by the sequence-specific binding activity." This element is not satisfied because the restriction endonuclease taught by Bloch et al. cleaves the nucleic acid molecules bearing a recognition sequence, rather than retaining them, and the result is not an enriched population.

In view of the above, Applicants submit that the combination of Bloch et al. and Oefner et al. does not result in the invention of claim 1 or claims 2-3 and 145-155 that depend from it.

Applicants submit that neither Bloch et al. nor Oefner et al. teaches or suggests, singularly or in combination, a method of enriching for and identifying nucleic acid sequence differences with respect to a reference sequence as recited in claim 57. This is because no combination of Oefner et al. and Bloch et al. can be construed as teaching the required elements of claim 57 in the order required by the claim language, as discussed above in relation to Oefner et al. Thus, even if Bloch et al. does teach or suggest the step of fragmenting the sample by

endonuclease digestion, it does not teach the order of steps required by claim 57 and lacking from Oefner et al. Bloch et al. does not teach or suggest an order of steps including a) fragmenting a nucleic acid sample from one or more individuals, b) physically separating a subset of nucleic acid fragments based on the size of the fragments, c) operatively linking the subset of step (b) with molecules capable of being replicated, d) introducing the linked subset of molecules of step (c) into a system capable of replicating the linked subset of molecules, and replicating the subset of linked molecules to form an enriched collection of replicated molecules, and e) detecting one or more nucleotide differences in the members of the collection of step (d) with a method capable of detecting one or more nucleotide differences with respect to a reference sequence. Because Bloch et al. does not remedy the deficiencies of Oefner et al. with respect to claim 57, the combination of Oefner et al. with Bloch et al. cannot be said to render obvious the invention of claim 57.

Applicants submit that independent claim 69 as amended requires "fragmenting a nucleic acid sample from one or more individuals." While Bloch et al. may teach the use of restriction endonucleases to generate samples to prove the principle of their claimed invention, a modification of HPLC elution buffer that increases the resolution of HPLC for nucleic acid analysis, Bloch et al. does not teach or suggest that the fragmentation with restriction endonucleases would be useful in a method of enriching for and identifying nucleic acid sequence differences with respect to a reference sequence. As discussed above, Oefner et al. does not teach or suggest fragmenting a sample as part of a method of enriching for and identifying nucleic acid sequence differences with respect to a reference sequence. Therefore, neither Oefner et al. nor Bloch et al. teaches or suggests a method meeting the requirements of claim 69 as amended. Applicants therefore submit that no combination of these two references can teach or suggest a method that entails that step in a method of enriching for and identifying nucleic acid sequence differences with respect to a reference sequence, as required by amended claim 69. Further, no combination of these references can therefore teach or suggest the methods of claims 70-74 that depend from amended claim 69.

In view of the above, Applicants submit that amended independent claims 1, 57 and 69 and claims 2-3, 145-155, 58-68 and 70-74 that depend from them are not obvious over the

combination of Oefner et al. and Bloch et al. Applicants therefore respectfully request withdrawal of this §103 rejection of these claims.

B) The Oefner et al. reference in view of Bloch et al. and Fox et al.:

Claims 1-3, 57-74 and 145-156 are rejected under §103(a) over Oefner et al. in view of Bloch et al. in further view of Fox et al. (U.S. 6,140,086). The Office Action states that Oefner et al. in view of Bloch et al. "does not teach the method wherein the infrequently cleaving restriction endonuclease is selected from NotI," and that Fox et al. teaches NotI as an infrequently cleaving restriction endonuclease. The Office Action concludes that it would have been obvious "to substitute and combine within the method of comparative hybridization and sequencing of Oefner et al. in view of Bloch et al., the method of NotI restriction endonuclease digestion of Fox et al. Applicants respectfully disagree.

First, Fox et al. does not teach Not I as an infrequently cleaving nuclease. The reference states that "Restriction endonucleases that may be advantageously used in the methods of the invention include, but are not limited to AluI, Eco47 III, EcoRV, FspI, HpaI, MscI, NruI, PvuII, RsaI, ScaI, SmaI, SspI, StuI, ThaI, AvaI, BamHI, BanII, BglII, ClaI, EcoRI, HindIII, HpaII, KpaI, MseI, NcoI, NdeI, NotI, PstI, RvuI, SacI/SstI, SalI, XbaI, XhoI and I-Ceu I." There is no teaching in Fox et al. that Not I or any other enzyme is useful because it cleaves infrequently. If this were to be inferred from the list of enzymes Fox provides, it is not consistent with the inclusion of AluI, RsaI, HpaII and MseI in the same list. Each of these enzymes recognizes a 4 base recognition site and therefore cleaves much more frequently than NotI. Further, none of AluI, RsaI, HpaII and MseI fall within the definition of "infrequent" cutting set forth in the present specification. Therefore, Fox et al. does not teach NotI as an infrequent cutter, nor does it teach any advantage of infrequent cutting.

Further, the disclosure of Fox et al. is not sufficient to remedy the deficiencies of the combination of Oefner et al. with Bloch et al., as described above. Specifically, with respect to independent claim 1 and claims that depend from it, Fox et al. does not teach or suggest that the sample comprises a subset of nucleic acid molecules having a sequence that binds the sequence specific binding activity wherein a bound subset of nucleic acid molecules is retained by the

sequence specific binding activity. This is because NotI restriction endonuclease *cleaves*, rather than *retains* those molecules having a recognition sequence.

With respect to independent claim 57, while the reference may teach the use of NotI to cleave DNA, Fox et al. does not teach or suggest a method of enriching for and identifying nucleic acid sequence differences with respect to a reference sequence comprising the recited steps in the order required by the claim language, as discussed above in reference to claim 57 in view of Oefner et al. and Bloch et al. Because Fox et al. does not remedy the deficiencies of Oefner et al. in view of Bloch et al. with respect to the order of steps recited in claim 57, no combination of the references can render obvious the claim or claims that depend from it.

With respect to claim 69 as amended, Applicants submit that the Fox et al. reference does not remedy the deficiencies of Oefner et al. in view of Bloch et al. As discussed above, the combination of Oefner et al. and Bloch et al. proposed by the Office Action does not teach or suggest a step of fragmenting a nucleic acid sample from one or more individuals, in a method of enriching for and identifying nucleic acid sequence differences with respect to a reference sequence. The Fox et al. reference may provide a teaching of NotI restriction endonuclease cleavage of a sample, but that teaching is in reference to a *cloning* method. That is, Fox et al. provides no teaching or suggestion that cleavage with NotI would be useful for fragmenting a nucleic acid sample from one or more individuals in a method of enriching for and identifying nucleic acid sequence differences with respect to a reference sequence. Because 1) the combination of Oefner et al. in view of Bloch et al. as proposed by the Office Action does not teach or suggest fragmenting in the context of a method of enriching for and identifying nucleic acid sequence differences with respect to a reference sequence, and 2) Fox et al. similarly provides no teaching or suggestion that cleavage with NotI would be useful for fragmenting a nucleic acid sample from one or more individuals in a method of enriching for and identifying nucleic acid sequence differences with respect to a reference sequence, Applicants submit that there is no motivation provided by Oefner et al., Bloch et al., or Fox et al. for one to combine Fox et al.'s teaching of NotI cleavage in a *cloning* method with the teachings of Oefner et al. and Bloch et al. to achieve a *method of enriching for and identifying nucleic acid sequence differences with respect to a reference sequence*. Therefore, Applicants submit that no combination of Fox et al. with Oefner et al. and Bloch et al. can render obvious the method of

claim 69 as amended. In view of this, Applicants submit that claims 70-74, which depend from claim 69, also cannot be obvious over this combination of references.

In view of the above discussion regarding the combination of Oefner et al., Bloch et al., and Fox et al., Applicants respectfully request withdrawal of this § 103(a) rejection of claims 1-3, 57-74 and 145-156.

In view of the above amendments and remarks, Applicants submit that all issues related to the patentability of the claims have been addressed. Applicants respectfully request reconsideration of the claims.

Respectfully submitted,

Date: 8/28/01

Name: Mark J. Fitzgerald 45,928 for
Kathleen Williams 34,380
Palmer & Dodge LLP
One Beacon Street
Boston, MA 02108
Tel: 617-573-0100

Version of claims marked to show changes:

1. (Amended) A method of enriching for and identifying a nucleic acid sequence difference with respect to a reference sequence comprising:

a) contacting a nucleic acid sample with a molecule comprising a sequence-specific binding activity under conditions which permit specific binding, wherein said sample comprises a subset of nucleic acid molecules having a sequence that binds to said sequence-specific binding activity, and wherein a bound subset of nucleic acid molecules is retained by the sequence-specific binding activity, such that the subset of bound nucleic acid molecules is enriched for molecules comprising the sequence recognized by the sequence specific binding activity; and

b) detecting a sequence difference with respect to a reference sequence in the subset of nucleic acid molecules[.],

<u>wherein steps (a) and (b) enrich for and identify a nucleic acid sequence difference with respect to a reference sequence.</u>

57. (Amended) A method of enriching for and identifying nucleic acid sequence differences with respect to a reference sequence comprising:

a) fragmenting a nucleic acid sample from one or more individuals;

b) physically separating a subset of said nucleic acid fragments based on the size of the fragments [.];

c) operatively linking said subset of step (b) with [molecules capable of being replicated] <u>an oligonucleotide or a vector</u>;

d) [introducing the linked subset of molecules of step (c) into a system capable of replicating said linked subset of molecules, and] replicating said <u>operatively linked</u> subset [of linked molecules] to form an enriched collection of replicated molecules; and

e) detecting one or more nucleotide sequence differences in the members of said collection of step (d) with a method [capable of detecting] that detects one or more nucleotide differences with respect to a reference sequence[.],

wherein steps (a)-(e) enrich for and identify a nucleic acid sequence difference with respect to a reference sequence.

69. (Amended) A method of enriching for and identifying nucleic acid sequence differences with respect to a reference sequence comprising:

a) fragmenting a nucleic acid sample from one or more individuals;

b) physically separating a subset of said nucleic acid fragments based on the size of the fragments;

c) detecting one or more nucleic acid sequence differences with respect to a reference sequence in the members of said separated molecules of step (b), [with a method capable of detecting one or more nucleotide differences with respect to a reference sequence]

wherein steps (a)-(c) enrich for and identify a nucleic acid sequence difference with respect to a reference sequence.

EXHIBIT A

QUICK REFERENCE GUIDE

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Average Fragment Size Generated by Endonuclease Cleavage

Enzyme	Sequence	ATH	CEL	DRO	ECO	HUM	MUS	RSS	YSC	STA	XEL
Apa I	GGGCCC	25,000	40,000	6,000	15,000	2,000	3,000	8,000	20,000	70,000	5,000
	GGCGCGCC	400,000	400,000	60,000	20,000		100,000	4,000,000	500,000	600,000	200,000
Avr II	CCTAGG	15,000	20,000	20,000	150,000	8,000	7,000	10,000	20,000	20,000	15,000
*Bam*H I	GGATCC	6,000	9,000	4,000	5,000	5,000	4,000	5,000	9,000	15,000	5,000
Bgl I	GCCN$_5$GGC	20,000	25,000	4,000	3,000	3,000	4,000	10,000	15,000	30,000	6,000
Bgl II	AGATCT	2,000	4,000	4,000	6,000	3,000	3,000	3,000	4,000	6,000	3,000
*Bss*H II	GCGCGC	50,000	30,000	6,000	2,000		15,000	80,000	30,000	40,000	20,000
Dra I	TTTAAA	2,000	1,000	1,000	2,000	2,000	3,000	2,000	1,000	1,000	2,000
Eag I	CGGCCG	10,000	20,000	3,000	4,000		15,000	60,000	20,000	50,000	15,000
*Eco*R I	GAATTC	4,000	2,000	4,000	5,000	5,000	5,000	3,000	3,000	4,000	4,000
Hind III	AAGCTT	1,000	3,000	4,000	5,000	4,000	3,000	6,000	3,000	2,000	3,000
Nae I	GCCGGC	6,000	15,000	3,000	2,000	4,000	6,000	40,000	15,000	20,000	6,000
Nar I	GGCGCC	10,000	15,000	3,000	2,000	4,000	6,000	50,000	15,000	15,000	7,000
Nhe I	GCTAGC	10,000	30,000	10,000	25,000		10,000	15,000	10,000	10,000	10,000
Not I	GCGGCCGC	200,000	600,000	30,000	200,000		200,000	2,000,000	450,000	1,000,000	200,000
Pac I	TTAATTAA	70,000	20,000	25,000	50,000		100,000	100,000	15,000	9,000	50,000
Pme I	GTTTAAAC	60,000	40,000	40,000	40,000		80,000	50,000	50,000	25,000	50,000
Rsr II	CGGWCCG	25,000	50,000	15,000	10,000		60,000	50,000	60,000	150,000	70,000
Sac I	GAGCTC	3,000	4,000	4,000	10,000	3,000	3,000	3,000	9,000	10,000	4,000
Sac II	CCGCGG	10,000	20,000	5,000	3,000	6000	8,000	70,000	20,000	40,000	15,000
Sal I	GTCGAC	6,000	8,000	5,000	5,000		20,000	25,000	10,000	15,000	15,000
Sbf I	CCTGCAGG	100,000	200,000	50,000	40,000		15,000	60,000	150,000	200,000	30,000
Sfi I	GGCCN$_5$GGCC	400,000	1,000,000	60,000	150,000		40,000	150,000	350,000	2,000,000	100,000
*Sgr*A I	CRCCGGYG	30,000	100,000	20,000	8,000		80,000	100,000	90,000	150,000	90,000
Sma I	CCCGGG	10,000	30,000	10,000	6,000	4,000	5,000	30,000	50,000	50,000	5,000
Spe I	ACTAGT	8,000	8,000	9,000	60,000		15,000	6,000	6,000	6,000	8,000
Sph I	GCATGC	10,000	15,000	5,000	4,000	6,000	6,000	7,000	10,000	9,000	6,000
Srf I	GCCCGGGC	400,000	1,000,000	90,000	50,000		90,000	1,000,000	600,000	2,000,000	100,000
Ssp I	AATATT	3,000	1,000	1,000	2,000	2,000	3,000	2,000	1,000	1,000	2,000
Swa I	ATTTAAAT	50,000	9,000	15,000	40,000		60,000	25,000	15,000	6,000	30,000
Xba I	TCTAGA	5,000	4,000	9,000	70,000	5,000	8,000	7,000	4,000	5,000	6,000
Xho I	CTCGAG	4,000	5,000	4,000	15,000	7,000	7,000	6,000	15,000	25,000	10,000

Average size fragments predicted for ATH Arabidopsis thaliana, CEL Caenorhabditis elegans, DRO Drosophila melanogaster, ECO Escherichia coli, HUM Human, MUS Mouse, RSS Rhodobacter sphaeroides, YSC Saccharomyces cerevisiae, STA Staphylococcus aureus, and XEL Xenopus laevis.

Digestion of Agarose-Embedded DNA

Pulsed field gel electrophoresis (PFG) techniques have made possible the resolution of DNA molecules up to several million base pairs in length (1,2). Manipulation of naked DNA of this size in liquid creates double-stranded breaks due to mechanical shear forces. To avoid this fragmentation, DNA can be embedded in an agarose matrix. Intact cells are immobilized in agarose, and then treated to disrupt their cell walls and remove cellular protein. Subsequently, the DNA-containing agarose plug is manipulated in much the same way as DNA in solution. Protocols can be found on our home page.

Restriction endonuclease digestion of agarose-embedded DNA has proved to be a valuable tool in the physical mapping of chromosomes (3,4). Most endonucleases can function in the presence of agarose (5). Since diffusion within agarose is more limited than in liquid reactions, higher concentrations of restriction endonucleases and longer incubation times are generally required for complete DNA digestion. The extended times, together with the larger size of the target DNA, increase the necessity for highly purified restriction endonucleases lacking contaminating nuclease activities.

Digestion Protocol for Agarose-Embedded DNA

1) Cut 10–20 µl (1x3x5 mm) from a plug of agarose-embedded DNA (1–2 µg) with a scalpel or razor blade and immerse in 1.0 ml of TE in a microcentrifuge tube. Chill on ice for 15–30 min, occasionally inverting the tube to ensure equilibration. (This wash lowers the EDTA concentration in the plug).

2) Carefully remove the TE with a 1.0 ml pipet tip and tap the tube to place the plug at the bottom. Add 100 µl of restriction endonuclease buffer and chill on ice for 15–30 minutes (this wash equilibrates the agarose-embedded DNA in endonuclease digestion conditions).

3) Replace the restriction endonuclease buffer with fresh buffer and add the appropriate restriction endonuclease. Incubate at the recommended reaction temperature.

   Note: *Higher enzyme concentrations and longer incubation times must be used to achieve complete DNA cleavage in agarose as compared to solution. As a general rule, use 20 units for a 4 hour incubation or 5 units for a 16 hour incubation. Most restriction endonucleases will not cut to completion in less than 2 hours, regardless of the number of units present (see page 278 for information about specific enzymes).*

4) Following the restriction endonuclease digestion, chill the tube on ice and aspirate the enzyme and buffer. Equilibrate the plug with 1.0 ml of PFG running buffer on ice for 15-30 minutes, inverting the tube occasionally.

5) Carefully aspirate all but 100 µl of the PFG running buffer from the tube and load the plug onto the running gel with a clean spatula.

6) Perform PFG according to the manufacturer's instructions for your equipment. Conditions for optimizing DNA separation by PFG have been published (6).

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